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Measuring Seven Endogenous Ketolic Estrogens Simultaneously in Human Urine by High-Performance Liquid Chromatography−Mass Spectrometry

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A rapid, sensitive, and specific high-performance liquid chromatography-electrospray ionization-multistage mass spectrometry (MS) method for measuring endogenous ketolic estrogen metabolites in human urine has been developed. The method requires a single hydrolysis/extraction/derivatization step and only 2.5 mL of urine, yet is able to simultaneously quantify estrone and its 2-methoxy and 2-, 4-, and 16α-hydroxy derivatives, 16-ketoestradiol, and 2-hydroxyestrone-3-methyl ether metabolites. The combination of a simple hydrazone derivatization step with multistage MS greatly enhances the sensitivity and specificity of the analysis of endogenous estrogen within human urine. Standard curves are linear over a 100-fold concentration range with linear regression correlation coefficients typically greater than 0.99. The lower limit of quantitation for each ketolic estrogen is 0.2 ng/2.5-mL urine sample (10 pg on column), with an accuracy of 93−103% and an overall precision, including the hydrolysis, extraction, and derivatization steps, of 1−13% relative standard derivation (RSD) for samples prepared concurrently and 8−16% RSD for samples prepared in separate batches. This method also allows for the identification of 2-hydroxyestrone-3-methyl ether in urine obtained from both pre- and postmenopausal women. This potentially protective estrogen metabolite has been previously reported only in the urine of pregnant women. Since individual patterns of estrogen metabolism may influence the risk of breast cancer, accurate and specific measurement of estrogen metabolites in biological matrixes will facilitate future research on breast cancer prevention, screening, and treatment.

The evidence that endogenous estrogens play an important role in the development of breast cancers is overwhelming.[1] Increased risk for breast cancer has been reported in women with high circulating and urinary estrogen levels, as well as in those exposed to increased estrogen levels over time as a result of early onset of menstruation, late menopause, postmenopausal obesity, and/or use of hormone replacement therapy after menopause.[2−4] There are two leading hypotheses regarding the role of estrogens in breast carcinogenesis. One of these hypotheses involves catechol estrogens, mainly 2-hydroxyestrone (2-OHE_1) and 4-hydroxyestrone (4-OHE_1) (Figure 1), which can form both stable and depurinating DNA adducts and lead to cell transformation and cancer initiation.[5] The formation of such guaiacol estrogens via catechol-*O*-methyltransferase-mediated methylation of catechol estrogens has been postulated as a key mechanism in deactivating catechol estrogens and inhibiting estrogen carcinogenesis.[6] Alternatively, it has been proposed that the potent mitogenic effects of estrogen are the key mechanisms leading to carcinogenesis. Under this hypothesis, the 16α-hydroxylated estrogens, such as 16α-hydroxyestrone (Figure 1), would be responsible for breast carcinogenesis due to their much stronger hormonal and mitogenic activity compared to the catechol estrogens.[7] It is conceivable that quantitatively comparing the levels of endogenous estrogen metabolites in women that ultimately develop breast cancer to those who remain healthy could help elucidate the mechanism of breast carcinogenesis and evaluate breast cancer risk in women.

Current methods for measuring endogenous estrogen metabolites have involved radioimmunoassay (RIA),[8−11] enzyme immu-

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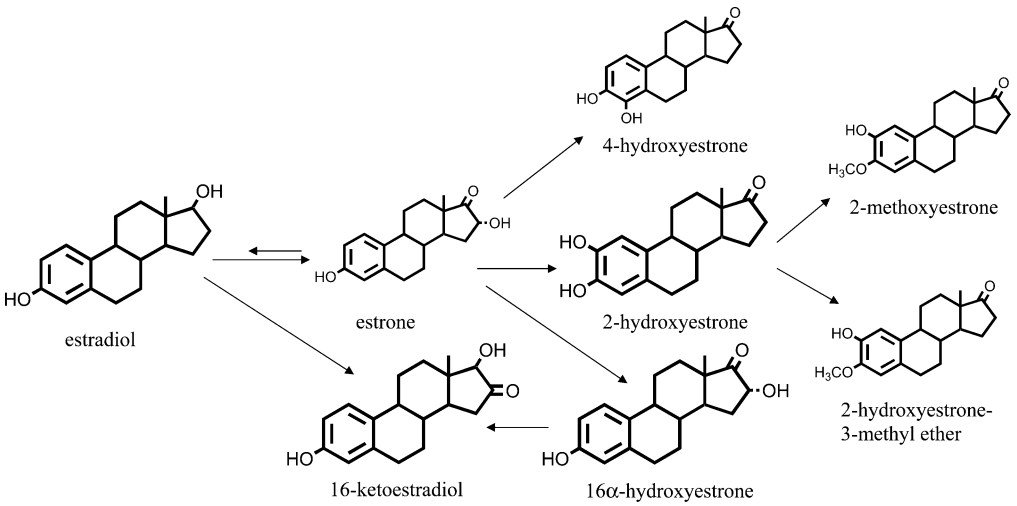

Figure 1. Formation of endogenous ketolic estrogen metabolites.

noassay (EIA),[12,13] high-performance liquid chromatography (HPLC) with electrochemical detection,[14−16] or stable isotope dilution combined with analysis using gas chromatography−mass spectrometry (GC/MS).[17] Although RIA and EIA can be sensitive and allow high throughput, they often suffer from poor specificity, accuracy, or reproducibility due to the cross-reactivity and lot-to-lot variation of antibodies.[18−21] Although HPLC with electrochemical detection has been used for estrogen metabolite analysis in hamsters treated with 17β-estradiol[22] and in pregnant women, whose estrogen levels are elevated at least 10-fold, it is relatively insensitive.[14−16] Stable isotope dilution combined with the GC/MS method is both sensitive and specific and has been successfully used not only for urine samples from nonpregnant premenopausal women but also for urine from postmenopausal women in which endogenous estrogen metabolites are substantially reduced.[17,23] Unfortunately this method is extremely labor intensive, requiring three solid-phase extractions, six ion-exchange column separations, four liquid−liquid extractions, and two derivatization procedures for each urine sample.[17,23] To overcome this problem, we recently developed a method using HPLC-electrospray ionization (ESI)-MS with selected ion monitoring for measuring endogenous 2-OHE_1 and 4-OHE_1 in pre- and postmenopausal urine.[24] That method, however, still requires three column extractions and 10 mL of urine/sample. In this report, we present an HPLC-ESI-MS^n method that requires single hydrolysis, extraction, and derivatization steps and only 2.5 mL of urine, yet has the sensitivity to quantitatively measure seven endogenous ketolic estrogens (Figure 1) found in urines from pre- and postmenopausal women, including both the catechol and the 16α-hydroxylated estrogen metabolites.

EXPERIMENTAL SECTION

Reagents and Materials. Seven ketolic estrogens (KE) including estrone (E_1), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 16-ketoestradiol (16-$KetoE_2$), and 16α-hydroxyestrone (16α-OHE_1) were obtained from Steraloids, Inc. (Newport, RI). Deuterium-labeled ketolic estrogens (d-KE), including estrone-2,4,16,16-d_4 (d_4-E_1), 2-hydroxyestrone-1,4,16,16-d_4 (d_4-2-OHE_1), 4-hydroxyestrone-1,2,16,16-d_4 (d_4-4-OHE_1), 2-methoxyestrone-1,4,16,16-d_4 (d_4-2-$MeOE_1$), and 16-ketoestradiol-2,4,15,15,17-d_5 (d_5-16-$KetoE_2$), were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, PQ, Canada). 16α-Hydroxyestrone-2,4,16-d_3 (d_3-16α-OHE_1) was synthesized by Dr. Victor Nelson in the Chemical Synthesis and Analysis Laboratory at NCI−Frederick (Frederick, MD). All KE and d-KE analytical standards have reported chemical and isotopic purity of ≥98%, and were used without further purification. *p*-Toluenesulfonhydrazide (TSH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanol (HPLC grade) and formic acid (reagent grade) were obtained from EM Science (Gibbstown, NJ). Water (HPLC grade) was obtained from Mallinckrodt Baker, Inc. (Paris, KY). Glacial acetic acid (HPLC grade) and L-ascorbic acid (reagent grade) were purchased from J. T. Baker (Phillipsburg, NJ), and sodium acetate (reagent grade) was purchased from Fisher Scientific (Fair Lawn, NJ). Diethyl ether (reagent grade) was obtained from VWR (West Chester, PA), and β-glucuronidase/sulfatase from *Helix pomatia* (type H-2) was obtained from Sigma Chemical Co. (St. Louis, MO).

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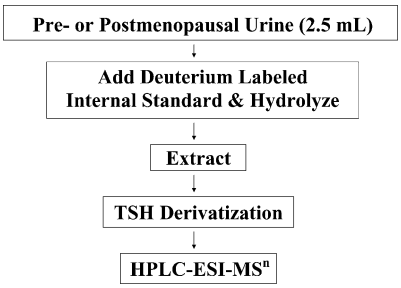

Figure 2. Summary of method for the analysis of ketolic estrogens in urine by HPLC-ESI-MS.

Urine Sample Collection. The 24-h urine samples were collected in 3-L bottles containing 3 g of ascorbic acid (to prevent oxidation) from two healthy nonpregnant premenopausal women (aged 34 and 38 years) and two healthy postmenopausal women (aged 58 and 60 years; >5 years past last menstrual cycle). None of the women was taking exogenous estrogens. The urine samples obtained from the two premenopausal women were collected during the midfollicular (days 8−9 of menstrual cycle) and midluteal (6 days before the anticipated menses) phases of the menstrual cycle. Immediately after collection, the volumes of the urine samples were recorded and sodium azide added to each sample to a final concentration of 0.1% (w/v). Since sodium azide in urine generates a toxic gas, it was added to the samples within a fumehood. Half of the 24-h urine samples from the two postmenopausal women were combined to prepare a pooled postmenopausal urine sample, and the remaining two fractions were kept separate as nonpooled postmenopausal urines. Similarly, pooled and nonpooled premenopausal urines during either midfollicular or midluteal phase were prepared. Aliquots of both pooled and nonpooled urines were stored at −80 °C prior to analysis.

Preparation of Stock and Working Standard Solutions. Stock solutions of KE and d-KE were each prepared at 80 μg/mL by dissolving 2 mg of the estrogen powders in methanol to a final volume of 25 mL in a volumetric flask and the resultant mixture was stored at −20 °C. During each day of analysis, working standards of KE and d-KE were prepared by serial dilutions of the stock solutions using 100% methanol. The d-KE working standard solution was prepared at 800 ng/mL, while KE working standard solutions were prepared at 80 and 8 ng/mL.

Preparation of Calibration Standards. Since KE are naturally present at various levels in all human urine samples and all seven KE were detected in male urine by our method, use of KE-spiked human urine to generate calibration curves was impractical. Therefore, calibration standards were prepared in methanol by combining 50 μL of the d-KE working internal standard solution (40 ng of d-KE) with various volumes of either KE working standard solution, which typically contained from 0.2 to 25.6 ng of KE.

Hydrolysis and Extraction Procedure. The overall procedure for the measurement of KE is shown schematically in Figure 2. Since endogenous estrogens and estrogen metabolites in urine are mostly present as glucuronide conjugates as well as small amounts of sulfate conjugates, a hydrolysis step was included. To a 2.5-mL aliquot of urine, 50 μL of the d-KE working internal standard solution (40 ng of d-KE) was added, followed by 2.5 mL of freshly prepared enzymatic hydrolysis buffer containing 10 mg of L-ascorbic acid, 25 μL of β-glucuronidase/sulfatase from *H. pomatia* (type H-2), and 2.5 mL of 0.15 M sodium acetate buffer (pH 4.1), as previously described.[17] The sample was incubated overnight at 37 °C. After hydrolysis, the sample was applied to an Extrelut QE column (EM Science, Gibbstown, NJ) and eluted with two 8-mL additions of diethyl ether.

Derivatization Procedure. The diethyl ether eluate containing both KE and d-KE was evaporated to dryness at 45 °C under nitrogen gas (Reacti-Vap III, Pierce, Rockford, IL). Both KE compounds were reacted with 400 μg *of p*-toluenesulfonhydrazide in 200 μL of methanol with 0.05 M acetic acid and heating at 60 °C (Reacti-Therm III Heating Module, Pierce) for 60 min to form the KE and d-KE *p*-toluenesulfonhydrazones (KE-TSH and d-KE-TSH, respectively). Calibration standard mixtures were derivatized in the same way. After derivatization, urine samples and calibration standards were evaporated to dryness at 60 °C under nitrogen and redissolved in 500 μL of methanol/water (1:1) for LC-ESI-MS analysis (Figure 2).

HPLC-ESI-MS^n. HPLC-ESI-MS analysis was performed on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer coupled with a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Both the HPLC and mass spectrometer were controlled by Xcalibur software (ThermoFinnigan). Liquid chromatography was carried out on a 150 mm long × 2.0 mm i.d. column packed with 3-μm Luna C18(2) particles (Phenomenex, Torrance, CA) maintained at 40 °C. A total of 25 μL of each processed urine sample was injected onto the column. The mobile phase consisted of methanol as solvent A and 0.1% (v/v) formic acid in water as solvent B. For the analysis of KE-TSH and d-KE-TSH, an isocratic gradient containing solvents A/B at a ratio of 60:40 for 30 min at a flow rate of 200 μL/min was employed. The divert valve was programmed to allow the chromatography effluent containing KE and d-KE to pass into the ESI interface for subsequent detection between 8 and 27 min of the isocratic HPLC fractionation. After washing with 100% A for 5 min, the column was reequilibrated with a mobile-phase composition A/B of 60:40 for 7 min prior to the next injection. Positive ionization mode was used with the following mass spectrometer parameters: ion source voltage, 5 kV; heated capillary temperature, 350 °C; capillary voltage, 7 V; sheath gas flow rate, 70 units; auxiliary gas flow rate, 15 units; tube lens offset, −15 V. MS full scan mode was employed for characterizing mass spectra of KE-TSH and d-KE-TSH. To perform MS/MS and MS^3; the m/z values of the ions of interest were input into the methods file used by the instrument during the LC−MS analysis. The experiment to be performed on each ion (i.e., MS/MS or MS^3) was also included. The instrument is fully capable of performing both MS/MS and MS^3 on selected ions within a single LC−MS analysis. Tandem MS (MS^2) full scan data for the protonated molecules [MH$^+$] of KE-TSH and d-KE-TSH were obtained at a relative collision energy of 35% as follows: 2-OHE$_1$, 4-OHE$_1$, 16-KetoE$_2$, and 16α-OHE$_1$ m/z 455 (453.5−456.5) → 125−500; 2-OHE$_1$-d$_4$ and 4-OHE$_1$-d$_4$ m/z 459 (457−461) → 125−500; 16-KetoE$_2$-d$_5$ m/z 460 (458−462) → 125−500; 16α-OHE$_1$-d$_3$ m/z 458 (457−460) → 125−500; E$_1$ m/z 439 (437.5−440.5) → 125−500; E$_1$-d$_4$ m/z 443 (441−445) → 125−500; 2-MeOE$_1$ and 3-MeOE$_1$ m/z 469 (467.5−470.5) → 125−500; 2-MeOE$_1$-d$_4$ m/z 473 (471−475) → 125−500. In addition, MS^3 full scan data for 16-KetoE$_2$

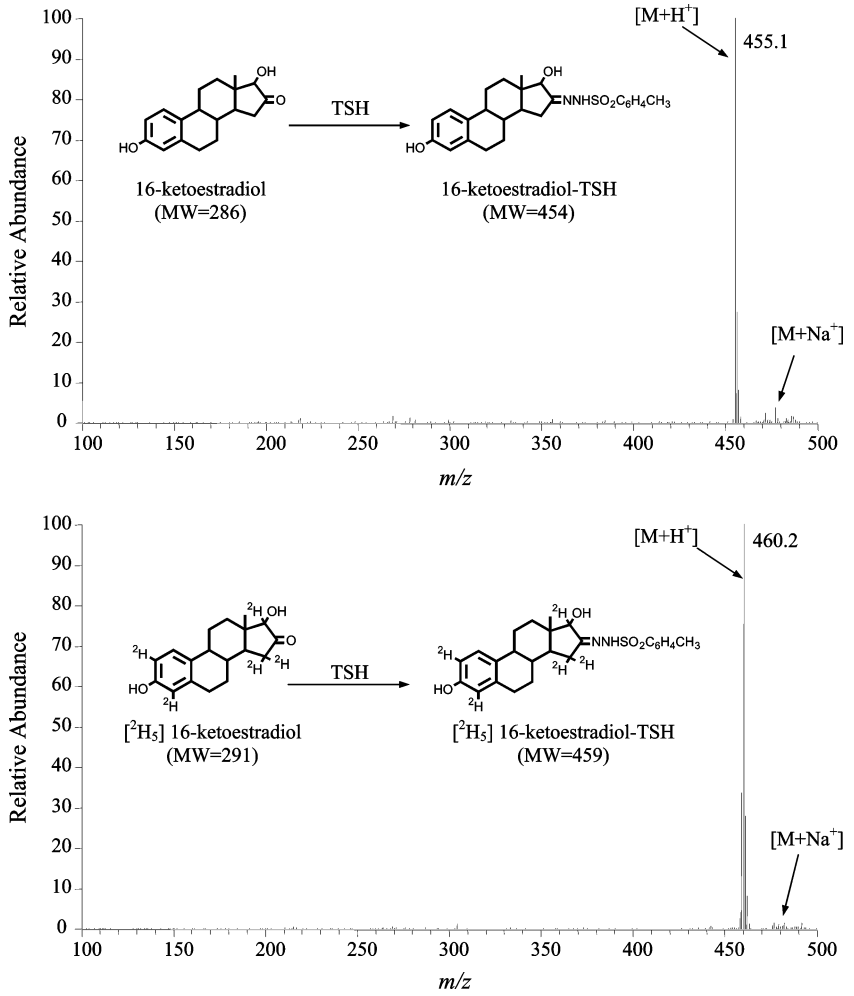

Figure 3. Example mass spectra for both KE-TSH and d-KE-TSH.

m/z 455 (453.5−456.5) → 299 (298−300) → 80−500 and 16-KetoE_2-d_5 m/z 460 (458−462) → 304 (302.5−305.5) → 80−500 were obtained.

Quantitation of KE. Quantitation of KE in urine was carried out with Xcalibur Quan Browser (ThermoFinnigan). Calibration curves for the six KE for which deuterated standards were available were constructed by plotting KE-TSH/d-KE-TSH peak area ratios obtained from calibration standards versus amounts of KE and fitting these data using linear regression with $1/X$ weighting. The amount of KE in urine samples was then interpolated using this linear function.

Exchange loss of deuterium was noted for d-KE-TSH during sample processing, analysis, or both. Therefore, the summed intensity of all observable [MH^+] ions in the d-KE-TSH cluster was isolated, and the summed intensity of their specific product ion cluster in MS^2 or MS/MS/MS (MS^3) full scan was used as the reference for quantitation. Specific product ions employed for quantitation from MS^2 full scan of the protonated molecules [MH^+] were as follows: m/z 173 (172.5−173.5) for 2-OHE_1 and 4-OHE_1; m/z 175 (174−176) for 2-OHE_1-d_4 and 4-OHE_1-d_4; m/z 257 (256.5−257.5) for 16α-OHE_1; m/z 260 (258.5−261.5) for 16α-OHE_1-d_3; m/z 157 (156.5−157.5) for E_1; m/z 159 (158−160) for E_1-d_4; m/z 187 (186.5−187.5) for 2-$MeOE_1$ and 3-$MeOE_1$; m/z 189 (188−190) for 2-$MeOE_1$-d_4. To avoid matrix contaminants, specific product ions from MS^3 full scan of 16-KetoE_2 and 16-KetoE_2-d_5, m/z 212.5−213.5, 240.5−241.5, 270.5−271.5 and m/z 214−216, 242−244, 275−277, respectively, were used for quantitation of these estrogen metabolites.

Assay for 2-Hydroxyestrone-3-methyl Ether. The identification of 3-$MeOE_1$ in both pre- and postmenopausal urine was based upon matching its chromatographic and mass spectral profiles with those obtained from the authentic standard. Because no deuterated standard was available, this metabolite was quantified using a procedure identical to that described in the previous section except that the calibration curve relied on d_4-2-$MeOE_1$as internal standard.

Absolute Recovery of KE after Hydrolysis and Extraction Procedure. To one set of six 2.5-mL aliquots of the pooled postmenopausal urine, 50 μL of the d-KE working internal standard solution (40 ng of d-KE) was added, followed by the hydrolysis and extraction procedure described above. A second set of six 2.5-mL aliquots of the pooled postmenopausal urine was treated identically, except that the d-KE was added *after* the hydrolysis and extraction procedure. Both sets of samples were derivatized and analyzed in consecutive LC−MS analyses. The absolute recovery of KE after the hydrolysis and extraction procedure was calculated by dividing the KE-TSH/d-KE-TSH peak area ratio from a sample of the first set into that from a sample of the second set and then calculating the mean of the six values.

Table 1. Summary of the Main Ions Observed during MS and MS^2 Full Scan of KE-TSH and d-KE-TSH, and MS^3 Full Scan of 16-Ketoe$_2$-TSH and [$^2H_5$] 16-Ketoe$_2$-TSH[a]

	MS full scan (m/z)	MS^2 full scan (m/z)	MS^3 full scan (m/z)
16-ketoE$_2$-TSH (MW=454.2)	455.1 (100)	299.1 (100)	213.1 (55) 240.9 (95) 271.1 (100)
2-OHE$_1$-TSH (MW=454.2)	455.2 (100)	173.2 (100) 269.0 (70)	
4-OHE$_1$-TSH (MW=454.2)	455.2 (100)	173.2 (100) 251.1 (25) 269.0 (90)	
16α −OHE$_1$-TSH (MW=454.2)	455.1 (100)	213.1 (40) 256.9 (45) 284.1 (40) 299.1 (100)	
E$_1$-TSH (MW=438.2)	439.2 (100)	157.2 (100) 253.0 (80)	
2-MeOE$_1$-TSH (MW=468.2)	469.2 (100)	187.2 (100) 283.1 (42)	
3-MeOE$_1$-TSH (MW=468.2)	469.1 (100)	187.2 (100) 283.1 (47)	
16-ketoE$_2$-d$_5$-TSH (MW=459.2)	458.2 (3) 459.2 (30) 460.2 (100)	303.1 (50) 304.1 (100)	215.1 (45) 243.0 (100) 275.1 (30) 276.1 (65)
2-OHE$_1$-d$_4$-TSH (MW=458.2)	457.3 (7) 458.2 (50) 459.2 (100)	174.2 (15) 175.2 (100) 271.1 (10) 272.1 (27) 273.1 (32)	
4-OHE$_1$-d$_4$-TSH (MW=458.2)	457.3 (6) 458.2 (53) 459.2 (100)	174.2 (15) 175.2 (100) 255.2 (12) 271.1 (13) 272.1 (32) 273.1 (47)	
16α −OHE$_1$-d$_3$-TSH (MW=457.2)	457.2 (12) 458.1 (100)	214.2 (20) 215.2 (75) 258.9 (25) 259.9 (65) 286.2 (15) 287.1 (7) 301.2 (30) 302.2 (100)	
E$_1$-d$_4$-TSH (MW=442.2)	441.3 (5) 442.3 (56) 443.2 (100)	158.2 (5) 159.3 (100) 255.1 (12) 256.1 (28) 257.1 (30)	
2-MeOE$_1$-d$_4$-TSH (MW=472.2)	471.3 (5) 472.3 (54) 473.2 (100)	188.2 (5) 189.2 (100) 285.1 (5) 286.1 (18) 287.1 (21)	

[a] Data are expressed as mass (relative abundance, %).

Accuracy and Precision of the Urinary KE Analysis. To assess accuracy and intrabatch precision, 50 μL of the d-KE working internal standard solution (40 ng of d-KE) was added to each of 18 2.5-mL aliquots of the pooled postmenopausal urine samples. Identical known amounts of KE (0, 2, or 8 ng, respectively) were added to each of the urine aliquots. All the urine samples were hydrolyzed, extracted, derivatized, and analyzed as described above. The endogenous KE concentration for the pooled postmenopausal urine was determined as the mean of the measured values for the six samples to which 0 ng of KE was added. This baseline KE concentration was then subtracted from the values determined for the 12 KE-spiked urine samples to assess method accuracy and intrabatch precision. In addition, duplicate aliquots of the pooled postmenopausal urine were hydrolyzed, extracted, derivatized, and analyzed in four different batches to assess the interbatch precision of the analysis. The intra- and interbatch precisions were measured as the relative standard deviations (RSD).

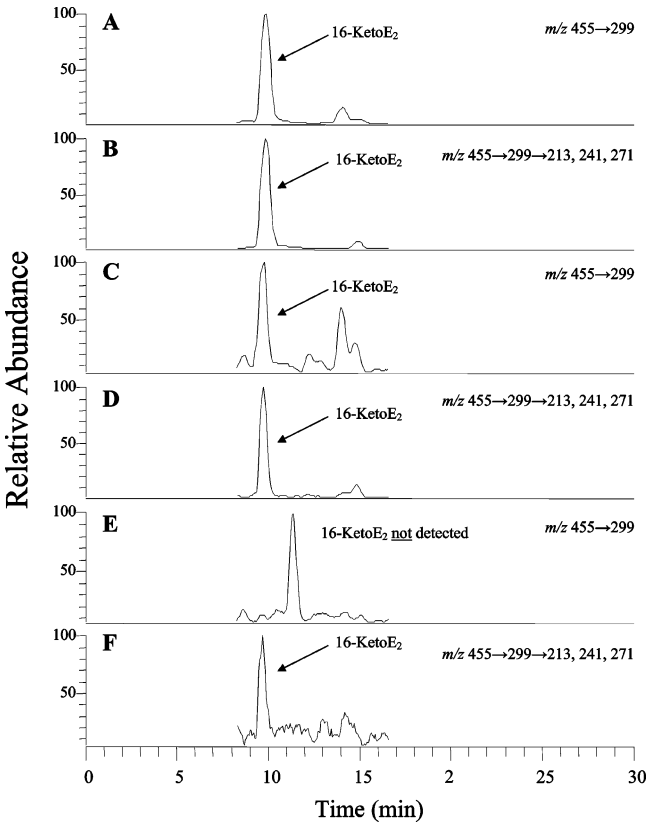

Figure 4. Selected ion chromatographic profiles: (A) MS^2 full scan of 16-Ketoe$_2$ standard; (B) MS^3 full scan of 16-Ketoe$_2$ standard; (C) MS^2 full scan of 16-Ketoe$_2$ in pooled premenopausal urine; (D) MS^3 full scan of 16-Ketoe$_2$ in pooled premenopausal urine; (E) MS^2 full scan of 16-Ketoe$_2$ in pooled postmenopausal urine; (F) MS^3 full scan of 16-Ketoe$_2$ in pooled postmenopausal urine.

RESULTS AND DISCUSSION

Mass Spectral and Chromatographic Profiles of Ketolic Estrogens in Standard and Pooled Human Urine. The mass spectra of KE-TSH and d-KE-TSH are characterized by an intense protonated molecule [MH^+] and a much less abundant sodiated molecule [MNa^+], less than 5% of [MH^+], as shown in Figure 3. In contrast to KE-TSH, the mass spectra of d-KE-TSH showed an isotopic contribution from different isotopomers: [$^2H_4$], [^2H_3H], and [$^2H_2H_2$] for [$^2H_4$]-estrone, [$^2H_4$]-2-hydroxyestrone, [$^2H_4$]-4-hydroxyestrone, or [$^2H_4$]-2-methoxyestrone; [$^2H_5$]-, [^2H_4H]-, and [$^2H_3H_2$]- for [$^2H_5$]-16-ketoestradiol; [$^2H_3$]- and [^2H_2H]- for [$^2H_3$]-16α-hydroxyestrone. This indicated that deuterium loss through back-exchange with protium had occurred during sample analysis (Table 1). To compensate for this back-exchange, the summed intensity of [$^2H_4$], [^2H_3H], and [$^2H_2H_2$], or [$^2H_5$], [^2H_4H], and [$^2H_3H_2$], or [$^2H_3$] and [^2H_2H] was isolated and the summed intensity of their specific product ion clusters in MS^2 or MS^3 full

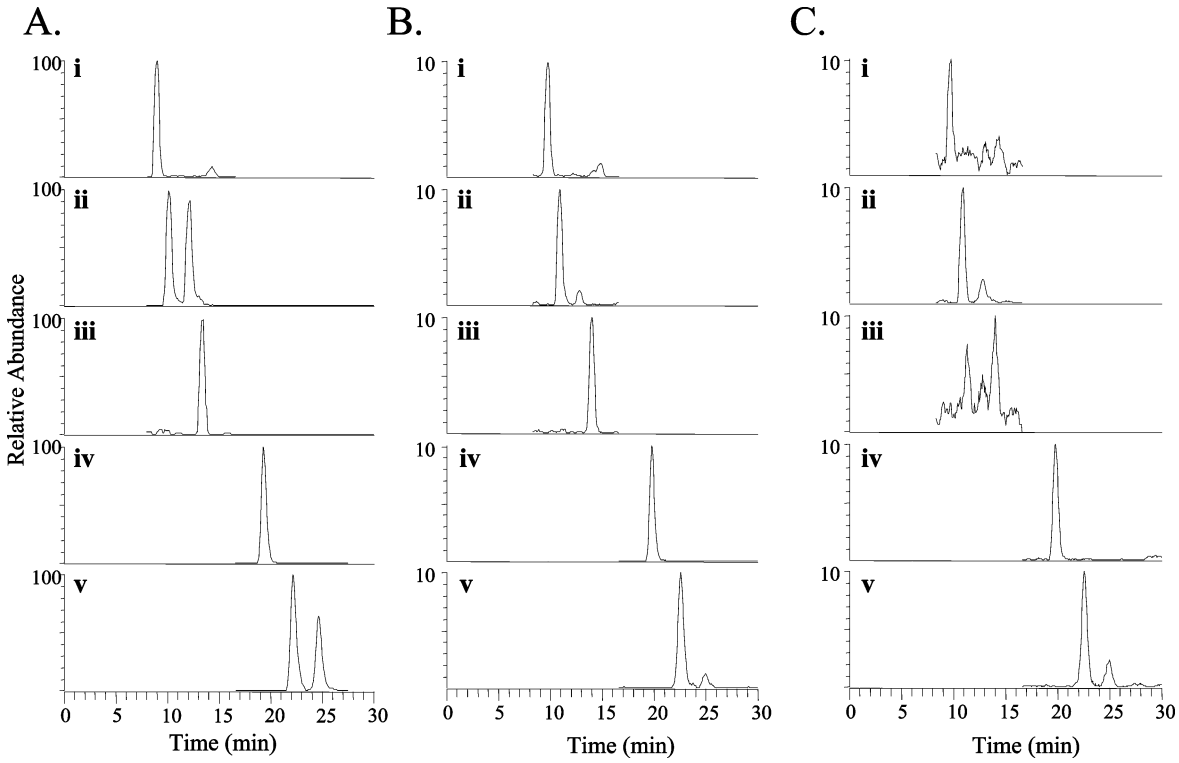

Figure 5. Chromatographic profiles of ketolic estrogens corresponding to (A) 0.2-ng working standard, (B) pooled premenopausal urine sample, and (C) pooled postmenopausal urine sample: (i) 16-KetoE$_2$-TSH, (ii) 2-OHE$_1$-TSH and 4-OHE$_1$-TSH, (iii) 16α-OHE$_1$-TSH, (iv) E$_1$-TSH, and (v) 2-MeOE$_1$-TSH and 3-MeOE$_1$-TSH.

scan was employed for d-KE-TSH for quantitative comparison with the corresponding KE-TSH peaks, as previously described.[24,25] The major ions observed in the ESI-MS, MS^2, and, where applicable, MS^3 mass spectra of the various KE analyzed in this study are listed in Table 1.

Although MS^2 full scan analysis is sufficient for analyzing most of the KE compounds in urine samples, it was necessary to analyze 16-KetoE$_2$ using a MS^3 full scan experiment to avoid matrix contaminants, especially in postmenopausal urine. The chromatographic profiles of the 16-KetoE$_2$ standard and of 16-KetoE$_2$ measured by MS^2 full scanning of the pooled pre- and postmenopausal urine samples are shown in Figure 4A, C, and E, respectively. While 16-KetoE$_2$ could be detected in the standard and pooled premenopausal urine samples, it was not detected in the pooled postmenopausal urine sample. Monitoring using a MS^3 full scan, however, allowed for 16-KetoE$_2$ in urine acquired from postmenopausal women to be readily detected (Figure 4F).

The HPLC-ESI-MS^n chromatographic profiles for a 0.2-ng working standard in methanol, a pooled premenopausal urine sample, and a pooled postmenopausal urine sample are shown in Figure 5A−C, respectively. Using a simple methanol/water isocratic gradient, the *p*-tosylhydrazones of all seven KE were separated by reversed-phase C$_{18}$ chromatography over a 30-min time range. All seven separated KE gave symmetrical peak shapes, which is essential for making accurate quantitative measurements. Even though only single hydrolysis, extraction, and derivatization steps and a 2.5-mL pre- or postmenopausal urine sample was used, this volume of urine was adequate to quantitatively analyze endogenous KE in human urines using this method.

HPLC−MS Identification of 2-Hydroxyestrone-3-methyl Ether in Human Urine. To confirm the identification of 3-MeOE$_1$ in pre- and postmenopausal urines, the chromatographic and mass spectral profiles of urine extracts were compared to those of a known standard. Chromatographic and mass spectral profiles of 3-MeOE$_1$ from pre- and postmenopausal urines matched well with those from the authentic standard (Figure 6), which confirmed its identification in the urine samples. Since the stable isotope-labeled 3-MeOE$_1$ was not available, d$_4$-2-MeOE$_1$ was employed as internal standard during quantitative analysis of urinary 3-MeOE$_1$.

Calibration Curve and Limit of Quantitation. An important consideration in the development of any assay is the linearity of the response and the sensitivity of the assay. The calibration curves for the detection of each KE were linear over a 100-fold range of concentration (0.2−25.6 ng/sample or 0.08−10.24 ng/mL) with correlation coefficients for the linear regression curves typically greater than 0.99. The signal-to-noise (S/N) ratios obtained for the 0.2-ng working standard, representing 10 pg of KE (37 fmol of E$_1$, 35 fmol of 2-OHE$_1$, 4-OHE$_1$, 16α-OHE$_1$, or 16-KetoE$_2$, 33 fmol of 2-MeOE$_1$ or 3-MeOE$_1$) on column, were typically greater than 15 (Figure 5), which provided an adequate lower limit of quantitation for endogenous KE analyses in urines from postmenopausal women.

Absolute Recovery of KE after the Hydrolysis and Extraction Procedure. The absolute recovery of KE after the hydrolysis and extraction procedure was determined by comparing chromatographic peak area ratios of KE-TSH/d-KE-TSH in pooled urine from postmenopausal women that had been spiked with d-KE before and after the hydrolysis and extraction procedure. Using this method, the mean absolute recoveries ranged from 81.1 to 88.3%.

(25) Adlercreutz, H.; Fotsis, T.; Bannwart, C.; Wahala, K.; Brunow, G.; Hase, T. *Clin. Chim. Acta* **1991**, *199*, 263−278.

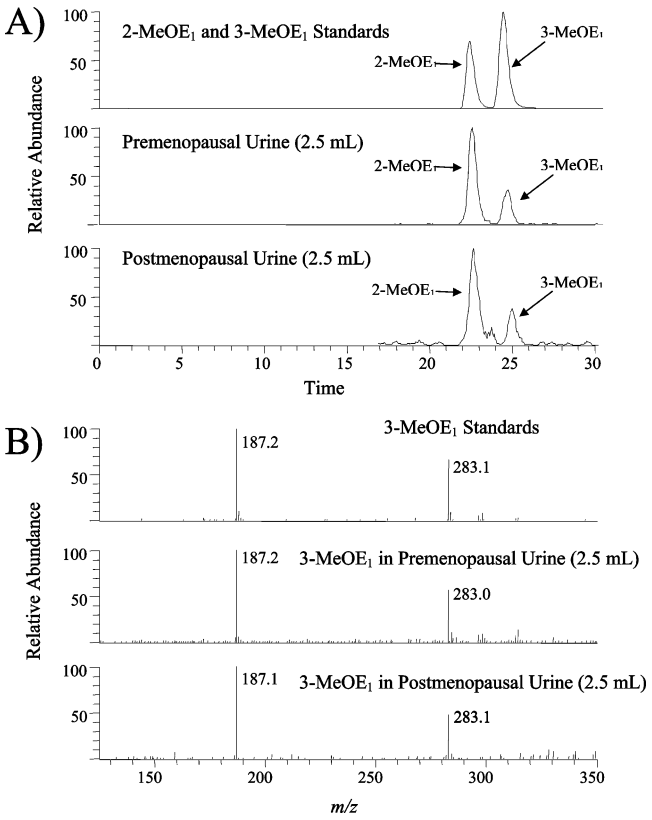

Figure 6. Identification of 2-hydroxyestrone-3-methyl ether in pre- and postmenopausal urines. The matching chromatographic and mass spectral profiles are shown in panels A and B, respectively.

Accuracy and Precision of the Urinary KE Analysis. Accuracy and intrabatch precision data for the stable isotope dilution HPLC-ESI-MS analysis of human urine samples are presented in Table 2. The analysis of six 2.5-mL aliquots of the pooled postmenopausal urine generated mean concentrations for each endogenous KE (Table 2). Subtraction of these baseline values from the mean KE concentrations of six identical pooled postmenopausal urine aliquots to which 2 or 8 ng of each KE had been added led to estimates of accuracy ranging from 93 to 103% and 98 to 102%, respectively (Table 2). The intrabatch precision, as estimated by the RSD from six replicate analyses at each level, was 5−14% for endogenous levels of urinary KE, 2−6% for urinary KE concentrations ranging from 2.2 to 4.8 ng/2.5 mL, and 1−6% for urinary KE concentrations ranging from 8.1 to 10.9 ng/2.5 mL, respectively (Table 2).

Interbatch precision data for the HPLC-ESI-MS analysis of human urine samples are presented in Table 3. The interbatch precision estimated by the RSD for four independent batch analyses of pooled postmenopausal urine samples ranged from 8 to 16% (Table 3).

Application to Pre- and Postmenopausal Urine Samples. The usefulness of this novel method to quantitate endogenous KE was demonstrated using six urine samples: two from postmenopausal women; two from premenopausal women in the follicular phase of their cycles; and two from the same premenopausal women in the luteal phase of their cycles. Duplicate 2.5-mL aliquots from each 24-h urine sample were hydrolyzed, extracted, derivatized, and analyzed to determine individual KE concentrations. The amounts of urinary KE excreted ranged from 4.6 to 19.7 nmol/24 h for 2-OHE_1, 0.7−2.6 nmol/24 h for 4-OHE_1, 4.2−13.9 nmol/24 h for 16-KetоE_2, 3.5−10.8 nmol/24 h for 16α-OHE_1, 6.2−26.1 nmol/24 h for E_1, 1.9−8.0 nmol/24 h for 2-$MeOE_1$, and 0.6−2.7 nmol/24 h for 3-$MeOE_1$, respectively (Figure 7). These results, in general, correspond very well with those previously reported for similar urine samples.[17,23,24,26] The method presented in this article, however, is sensitive enough to identify and quantitate 3-$MeOE_1$ in both pre- and postmenopausal urine samples. The detection of 3-$MeOE_1$ has previously been reported only in urine from pregnant women whose estrogen levels are elevated at least 10-fold compared to nonpregnant women.[27]

Table 2. Accuracy and Intrabatch Precision of Pooled, Postmenopausal Urinary Ketolic Estrogen Metabolite Analyses, Including Hydrolysis, Extraction, and Derivatization Steps[a]

	mean ($n = 6$)	SD ($n = 6$)	accuracy (%)	precision (% RSD)
urine sample + 0 ng				
16-ketоE_2	2.08	0.19	n/a[b]	9.2
2-OHE_1	2.29	0.17	n/a	7.4
4-OHE_1	0.41	0.03	n/a	7.7
16α-OHE_1	1.73	0.16	n/a	9.1
E_1	2.87	0.20	n/a	6.8
2-$MeOE_1$	0.98	0.05	n/a	5.0
3-$MeOE_1$	0.33	0.04	n/a	13.5
urine sample + 2 ng				
16-ketоE_2	4.04	0.21	98.1	5.2
2-OHE_1	4.28	0.19	99.6	4.5
4-OHE_1	2.44	0.10	101.6	4.1
16α −OHE_1	3.62	0.18	94.5	5.0
E_1	4.82	0.26	97.4	5.5
2-$MeOE_1$	3.04	0.05	102.9	1.7
3-$MeOE_1$	2.19	0.11	92.8	5.2
urine sample + 8 ng				
16-ketоE_2	10.07	0.25	99.9	2.4
2-OHE_1	10.14	0.21	98.2	2.1
4-OHE_1	8.25	0.19	98.0	2.3
16α −OHE_1	9.91	0.46	102.3	4.7
E_1	10.90	0.31	100.4	2.9
2-$MeOE_1$	9.10	0.10	101.4	1.1
3-$MeOE_1$	8.14	0.52	97.6	6.4

[a] The mean is expressed in units of ng/2.5 mL of urine. [b] n/a = not applicable.

Table 3. Interbatch Precision of Urinary Ketolic Estrogen Metabolite Analyses, including Hydrolysis, Extraction, and Derivatization Steps[a]

	mean ($n = 4$)	SD ($n = 4$)	precision (% RSD)
16-ketоE_2	2.29	0.23	10.1
2-OHE_1	2.39	0.20	8.3
4-OHE_1	0.50	0.06	12.5
16α −OHE_1	1.98	0.28	14.3
E_1	3.08	0.27	8.7
2-$MeOE_1$	1.03	0.08	8.2
3-$MeOE_1$	0.35	0.06	15.7

[a] The mean is expressed in units of ng/2.5 mL of urine.

(26) Xu, X.; Duncan, A. M.; Merz-Demlow, B. E.; Phipps, W. R.; Kurzer, M. S. *J. Clin. Endocrinol. Metab.* **1999**, *84*, 3914−3918.
(27) Saegusa, K., Suzuki, E., Anjo, T., Matsuki, Y., Nambara, T. *Biomed. Chromatogr.* **1993**, *7*, 172−176.

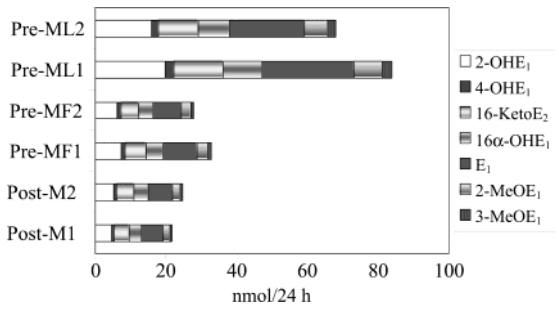

Figure 7. Urinary endogenous KE excretion (nmol/24 h) in postmenopausal women (Post-M) and premenopausal women during midfollicular (Pre-MF) and midluteal (Pre-ML) phases.

CONCLUSIONS

With mounting evidence that endogenous estrogens play a role in the development of breast cancer and that women with high circulating and urinary estrogen levels are at an increased risk,[1−4] it is important to develop a sensitive, accurate, and high-throughput assay that can measure individual endogenous estrogens in various biological fluids. Such an assay would facilitate epidemiologic and clinical research on how estrogen exposure and estrogen metabolism promote breast carcinogenesis. In addition, such an assay might contribute to predicting an individual woman's risk of developing breast cancer. Unfortunately, none of the currently published methods to measure endogenous estrogens combine accuracy, precision, and sensitivity with low cost, speed, and simplicity.

As a step toward meeting this need, we have developed a rapid, sensitive, specific, and accurate HPLC-ESI-MS method for measuring endogenous KE in human urine. Compared to the stable isotope dilution-GC/MS method, our approach greatly simplifies the sample preparation required for each sample resulting in an increased analytical throughput. The unique part of our method is the use of a simple hydrazone derivatization and multistage MS, which greatly improved the sensitivity and specificity of endogenous estrogen analysis in human urine samples and allowed us to identify 3-MeOE$_1$ in both pre- and postmenopausal women. Standard curves were linear over a 100-fold calibration range (0.2−25.6 ng of KE/sample), with linear regression correlation coefficients typically greater than 0.99. The lower limit of quantitation for each KE is 0.2 ng/2.5-mL urine sample, with an accuracy of 93−103% and an overall precision of 1−13% for samples prepared concurrently and 8−16% for samples prepared in several batches. This method is adequate for measuring the very low levels of endogenous KE in postmenopausal urines with high accuracy and sensitivity and is currently being evaluated within our laboratory to determine if there is a link between KE levels and breast cancer risk.

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